Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

CIRRHOSIS AND LIVER FAILURE

# Keratin-18 and microRNA-122 complement alanine aminotransferase as novel safety biomarkers for drug-induced liver injury in two human cohorts

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**Keywords**
acetaminophen – biomarkers – drug development – hepatotoxicity – HIV – liver – plasma – safety – toxicology – tuberculosis

**Abbreviations**
AFP, alpha-foetoprotein; ALT, alanine aminotransferase; DILI, drug-induced liver injury; ELISA, enzyme-linked immunosorbent assay; GLDH, glutamate dehydrogenase; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; K18, keratin 18; miR-122, microRNA-122; qRT-PCR, real-time quantitative reverse-transcription PCR; TB, tuberculosis.

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Received 19 June 2013
Accepted 31 August 2013

DOI:10.1111/liv.12322
Liver Int. 2014: 34: 367–378

## Abstract

*Background & Aims:* There is a demand for more sensitive, specific and predictive biomarkers for drug-induced liver injury (DILI) than the gold standard used today, alanine aminotransferase (ALT). The aim of this study was to qualify novel DILI biomarkers (keratin-18 markers M65/M30, microRNA-122, glutamate dehydrogenase and alpha-foetoprotein) in human DILI. *Methods:* Levels of the novel biomarkers were measured by enzyme-linked immunosorbent assay or real-time quantitative reverse-transcription PCR (qRT-PCR) in two human DILI cohorts: a human volunteer study with acetaminophen and a human immunodeficiency virus (HIV)/tuberculosis (TB) study. *Results:* In the acetaminophen study, serum M65 and microRNA-122 levels were significantly increased at an earlier time point than ALT. Furthermore, the maximal elevation of M65 and microRNA-122 exceeded the increase in ALT. In the HIV/TB study, all the analysed novel biomarkers increased after 1 week of treatment. In contrast to ALT, the novel biomarkers remained stable in a human cohort with exercise-induced muscular injury. *Conclusions:* M65 and microRNA-122 are potential biomarkers of DILI superior to ALT with respect to sensitivity and specificity.

Drug-induced liver injury (DILI) is the leading cause of acute liver failure, it is an important safety issue during drug development, and has been the most frequent single cause of safety-related drug marketing withdrawals (1, 2). Serum alanine aminotransferase (ALT) is used for detection of liver injury since the introduction into clinical monitoring some 50 years ago (3). ALT is a metabolic enzyme enriched in the

liver and hepatocellular injury is indicated by a rise of ALT in serum, reflecting release of ALT from injured cells (1). Elevated serum ALT levels are highly associated with features of liver injury such as hepatic steatosis, necrosis and inflammation. However, around 40% of human DILI cases are not detected in preclinical studies, which is why new more sensitive and mechanism-specific biomarkers are needed (4). There are several reasons why ALT is not an ideal biomarker for liver injury. It is not specific for the liver but also kidney, heart, skeletal muscle and pancreas possess ALT activity to a high degree (5). Hence, increased plasma ALT can result from muscular damage following exercise or subsequent to a myocardial infarction (6, 7). Also, enzymatic induction of the ALT gene is shown to occur during metabolic perturbations such as starvation, diabetes mellitus or during treatment with drugs that alter the metabolism. Thus, serum ALT might be elevated as a result of increased hepatocellular contents of ALT, which is released during normal hepatocyte turn-over, resulting in false positive signals for conditions separated from liver injury (8–10). In addition, ALT does not always correlate well with preclinical histopathological data, which results in problems in the interpretation of human clinical data, since liver tissue usually is not available (11). Therefore, additional biomarkers bridging between preclinical and clinical studies are needed. Biomarkers predicting DILI at an earlier stage than ALT, as well as diagnostic markers identifying treatment responses in patients would be helpful tools for clinical diagnosis. Thus, discovery and qualification of new DILI biomarkers, that give mechanistic insight and allow for prediction of DILI is an important goal for this research (12).

Candidates recently described as putative translational DILI biomarkers are keratin 18 (K18), microRNA-122 (miR-122), glutamate dehydrogenase (GLDH) and alpha-foetoprotein (AFP) (13–18). K18 is an intermediate filament protein, which is abundant in the liver (19). The full-length variant of the protein is released from necrotic cells, whereas the caspase-cleaved form of K18 arises from cells undergoing apoptotic cell death. Total plasma K18 can be detected by the epitope M65, present both on the full-length and cleaved form of K18 (necrosis and apoptosis), whereas the neoepitope M30 is formed on the K18 fragments during apoptotic cleavage. Circulating microRNAs have emerged as promising biomarkers because of their abundance and stability in biofluids and miR-122 is specific for the liver (20, 21). GLDH is a relatively liver-specific enzyme located in the mitochondrial matrix of hepatocytes and is considered to be a marker of necrosis (22). Following acute liver injury with extensive necrosis, an increase in serum AFP is interpreted as a sign of hepatic regeneration (23, 24).

Acetaminophen is a widely used analgesic that is safe at therapeutic doses (25), however, hepatotoxicity caused by acetaminophen overdose is the most frequent cause of acute liver failure in the western world today (26). Within this study, the potential novel DILI biomarkers K18 (M65 and M30), miR-122, GLDH and AFP are investigated regarding their sensitivity (with respect to time of elevation) to report liver injury in two different human DILI cohorts, caused by acetaminophen or HAART/anti-TB drugs (highly active antiretroviral therapy/antituberculosis), another leading cause of DILI (27–29) compared to currently used indicators such as ALT activity. Furthermore, the specificity (with respect to organ origin) of these biomarkers as markers of liver injury is also investigated in a human study of muscular injury.

## Materials and methods

### Acetaminophen study

This study has been described elsewhere (30). Analysis of serum samples for days 1–14 was performed for all novel biomarkers, except for miR-122 where only days 2–14 were analysed because of practical reasons. Written informed consent was obtained and approved by the UNC Institutional Review Board. None of the participants had any known history of liver disease.

### Human immunodeficiency virus/tuberculosis study

In Addis Ababa, Ethiopia, patients infected with human immunodeficiency virus (HIV) and/or TB were treated with HAART and/or anti-TB according to the national TB/HIV treatment guideline, as described (31). Blood was collected in heparin tubes at baseline and 1, 2, 4, 6, 8 and 12 weeks after the treatment was initialized. Written informed consent was obtained from each participant before the start of the study. This study protocol was approved by the Regional Ethical Review Board at Karolinska Institutet, Stockholm, Sweden; Institutional Review Board at Faculty of Medicine, Addis Ababa University; The National Ethics Review Committee at the Ethiopian Science and Technology Ministry; and the Food, Medicine and Health Care Administration and Control Authority of Ethiopia. None of the participants were positive for hepatitis B and only one was infected with hepatitis C at baseline and was hence not included in the miR-122 analysis.

### Muscular injury study

This study has been described elsewhere and was then referred to as the extreme adventure race study (17). For this study, a subset of 12 pre- and post-race plasma samples from the 48 h extreme adventure race were analysed for liver biomarkers. This study protocol was approved by the Regional Ethics Review Board in Stockholm, Sweden. None of the participants had any known history of liver disease.

### Enzymatic assays

Alanine aminotransferase activity in serum samples was measured in the three studies with pyridoxal phosphate activation using Cobas 501 instrument (acc. IFCC, ALT-LP: ACN 684; Roche Diagnostics, Mannheim, Germany). Upper limit of normal ALT activities from healthy individuals has in a large study been shown to be <37.5 U/L (32). The clinical chemistry markers aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin were measured in the three studies according to standard protocols. Creatine kinase (CK), γ-glutamyltransferase (GGT) and GLDH were only analysed in the HIV/TB and muscular injury studies, whereas creatinine, prothrombin time and activated partial thromboplastin time were measured in the acetaminophen study alone.

### Enzyme-linked immunosorbent assay

Total and caspase-cleaved K18 were determined in all three studies using the M65 (EpiDeath) and M30 (Apoptosense) enzyme-linked immunosorbent assay, respectively, in accordance with the manufacturer's guidelines (both assays from Peviva, Bromma, Sweden). One individual was excluded from the M65 results because of high baseline values. AFP was analysed in the HIV/TB study according to the manufacturer's instructions (R&D systems, Abingdon, UK). One patient with high baseline AFP levels (>30 ng/ml) was excluded from the analysis. In the statistical analysis, these two observations would exhibit a large degree of influence on the parameter estimates. To reduce the risk of distorting the statistical inference, it was therefore decided to exclude these two observations from the statistical analysis.

### Quantification of microRNA-122

#### *RNA extraction*

Total RNA, including miRNA was extracted from 50 μl of serum (acetaminophen study) or 100 μl heparin plasma (HIV/TB and muscular injury studies), using the miRNeasy-96 RNA isolation kit (Qiagen, Sollentuna, Sweden) following the manufacturer's instructions, with minor modifications according to the miRNeasy protocol for serum/plasma samples.

#### *Heparinase I treatment*

The RNA obtained from the HIV/TB and muscular injury studies was treated with heparinase I (Sigma-Aldrich, Schnelldorf, Germany) to remove contaminating heparin, based on the protocol described by Wang *et al.* (33). Ten microlitre of total RNA was incubated as 20 μl reactions containing 2 U heparinase I (Sigma-Aldrich) and 10 U RNAse inhibitor (Ambion/Life Technologies,Stockholm, Sweden) in 1× DNase I reaction buffer (Invitrogen/Life Technologies, Stockholm, Sweden) at 25°C for 1 h. Five microlitre of heparinase-treated RNA was subsequently used in the RT-reactions.

#### *Real-time quantitative reverse-transcription PCR analysis*

The expression levels of hsa-miRNA-122 were determined using the TaqMan miRNA reverse transcription kit and miRNA specific stem-loop primers (Applied Biosystems/Life Technologies, Stockholm, Sweden) according to the manufacturer's protocol. The miRNA was quantified on a 7900HT instrument (Applied Biosystems) using absolute quantification to a standard curve of synthetic RNA oligos (Sigma-Aldrich), included in the reverse transcription reaction. The quantities were normalized to the spike-in control *c-elegans* miR-39 (cel-39) (Qiagen).

### Statistical analysis

Descriptive statistics for liver injury markers were expressed as median and interquartile range. For the acetaminophen and HIV/TB studies, the liver markers were evaluated using a repeated measurement model. To meet the requirement of approximately normally distributed residuals, the data were log-transformed before the analysis. The model was fit using PROC MIXED in SAS V9.3 (SAS Institute Inc., Cary, NC, USA). Based on the parameter estimates, the relative change from baseline was calculated together with corresponding 95% confidence limits. The main endpoint for the evaluated biomarkers was the first time point where a significant change could be observed. Because of the absence of an independent reference for DILI, classical ROC curves could not be utilized. GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) was used to calculate Pearson correlation coefficients. To obtain approximate bivariate normality, the variables were also in this case log-transformed before the analysis. In the muscular injury study, the Wilcoxon matched pairs test was used to investigate whether there was a statistically significant difference between pre- and post-race samples.

## Results

### Biomarker qualification using a cohort of subjects exposed to acetaminophen

In a clinical study described by Winnike *et al.* (30), 58 healthy men and women were hospitalized and given a controlled whole-food diet (study days 1–3) before they received the maximum recommended therapeutic dose of acetaminophen: 4 g/day, for 7 days (study days 4–11). Blood was collected from all subjects from day 1 until day 14. Two groups were selected from this material: one group of 15 subjects who showed ALT elevations larger than two times their own baseline value and one group of 15 where ALT levels never increased more than 1.5 times from baseline levels. Clinical data for the

**Table 1.** Median values of liver injury markers in the 30 subjects in the acetaminophen study

| Markers | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | Day 8 | Day 9 | Day 10 | Day 11 | Day 12 | Day 13 | Day 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ALT (U/L) | Mdn | 30 | 30 | 31 | 33 | 31 | 32 | 33 | 36 | 43 | 52 | 57 | 52 | 50 | 48 |
| | IQR | 26–34 | 25–36 | 24–37 | 25–38 | 24–36 | 28–38 | 28–39 | 32–53 | 33–70 | 32–83 | 34–86 | 38–86 | 36–88 | 37–77 |
| AST (U/L) | Mdn | 24 | 23 | 24 | 26 | 24 | 25 | 25 | 29 | 33 | 31 | 33 | 30 | 34 | 30 |
| | IQR | 22–28 | 21–27 | 21–26 | 21–29 | 19–27 | 19–28 | 22–30 | 24–41 | 25–49 | 25–57 | 26–69 | 25–62 | 25–59 | 24–47 |
| ALP (U/L) | Mdn | 71 | 67 | 66 | 68 | 62 | 65 | 60 | 61 | 62 | 63 | 63 | 62 | 66 | 67 |
| | IQR | 61–80 | 57–83 | 57–79 | 58–84 | 56–78 | 56–76 | 55–76 | 54–74 | 54–73 | 55–73 | 56–75 | 55–72 | 57–75 | 58–75 |
| TBIL (μmol/L) | Mdn | 7 | 7 | 7 | 7 | 7 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 7 |
| | IQR | 5–9 | 5–10 | 4–9 | 5–10 | 3–7 | 4–9 | 3–7 | 3–7 | 3–7 | 4–8 | 5–7 | 3–7 | 3–9 | 5–9 |
| CR (mg/dl) | Mdn | 0.9 | 0.9 | 0.9 | 0.9 | 0.9 | 0.9 | 0.9 | 0.8 | 0.8 | 0.8 | 0.8 | 0.9 | 0.9 | 0.9 |
| | IQR | 0.8–1.0 | 0.8–1.0 | 0.8–1.0 | 0.7–1.0 | 0.7–0.9 | 0.7–1.0 | 0.8–1.0 | 0.8–1.0 | 0.7–1.0 | 0.8–1.0 | 0.8–1.0 | 0.8–1.0 | 0.8–1.0 | 0.8–1.0 |
| PT (s) | Mdn | 11 | – | – | – | 11 | – | – | – | – | 11 | – | – | – | 11 |
| | IQR | 10–12 | – | – | – | 11–12 | – | – | – | – | 10–12 | – | – | – | 10–12 |
| APTT (s) | Mdn | 32 | – | – | – | 33 | – | – | – | – | 34 | – | – | – | 33 |
| | IQR | 30–35 | – | – | – | 31–35 | – | – | – | – | 30–35 | – | – | – | 29–34 |

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TBIL, total bilirubin; CR, creatinine; PT, prothrombin time; APTT, activated partial thromboplastin time; Mdn, median; IQR, interquartile range.

30 subjects are shown in Table 1. The fold change values for the biomarkers evaluated in the study compared to baseline (defined as the average of day 1–3 for ALT, M65 and M30, and the average of day 2–3 for miR-122) are shown in Figure 1. ALT levels started to increase from day 8 (1.3-fold, $P < 0.001$) and onwards and reached its peak at study day 12 (1.9-fold, $P < 0.001$). ALT levels then stayed elevated in the remaining study period (Fig. 1a). The K18 markers M65 and M30 increased significantly from day 7 and 8 respectively (Fig. 1b,c). A larger fold change increase from baseline was observed for M65 compared to M30: at study day 8, M65 was elevated by 2.0-fold ($P < 0.001$) compared to baseline, whereas M30 only was marginally increased by 1.1-fold ($P = 0.04$). The K18 biomarkers both reached their maximal increases compared to baseline at day 11 (M65: 2.4-fold, $P < 0.001$; M30 1.3-fold, $P < 0.001$) and then declined after acetaminophen treatment was withdrawn. Analysis of serum miR-122 showed a significant increase from study day 7 and onwards (Fig. 1d). The relative fold change for miR-122 at study day 8 was as high as 2.2-fold ($P < 0.001$) and it was the biomarker reaching the maximal fold change compared to baseline (2.9-fold, $P < 0.001$). Pearson correlation coefficients between ALT and each of the novel potential biomarkers were calculated for all time points in the study and the correlations with ALT were significant from day 8 until the end of the study for all the biomarkers (Table 2). The strongest correlation with ALT was attained at day 11 with a correlation of 0.79 for M65, 0.69 for M30 and 0.71 for miR-122 respectively.

### Biomarker qualification using a human immunodeficiency virus/tuberculosis treatment cohort

In the HIV/TB study, patients treated for HIV and/or TB with potentially hepatotoxic drugs were followed up for 12 weeks after the commencement of treatment. Thirty-eight patients with ALT values exceeding three times their own baseline levels (elevation >30 U/L) at any time point during the study period were available for analysis. These patients were matched against 38 patients from the same treatment groups that did not show strong ALT elevations. Clinical chemistry data for the 76 individuals are found in Table 3 and the mean fold change values for the biomarkers investigated during the 12 weeks treatment period, compared to untreated patients at week 0, are found in Figure 2. ALT was increased from week 1 (1.3-fold, $P < 0.001$) and remained elevated at all time points in the study compared to baseline. The largest relative increase in ALT was observed at week two (1.5-fold, $P < 0.001$) (Fig. 2a). The K18 markers M65 and M30 were both significantly elevated at week 1 (M65:1.4-fold, $P < 0.001$; M30:1.1-fold, $P = 0.008$), but were not increased at any other time point in the study (Fig. 2b, c). miR-122 was analysed in a subset of 44 individuals; 22 with ALT elevations exceeding three times their own

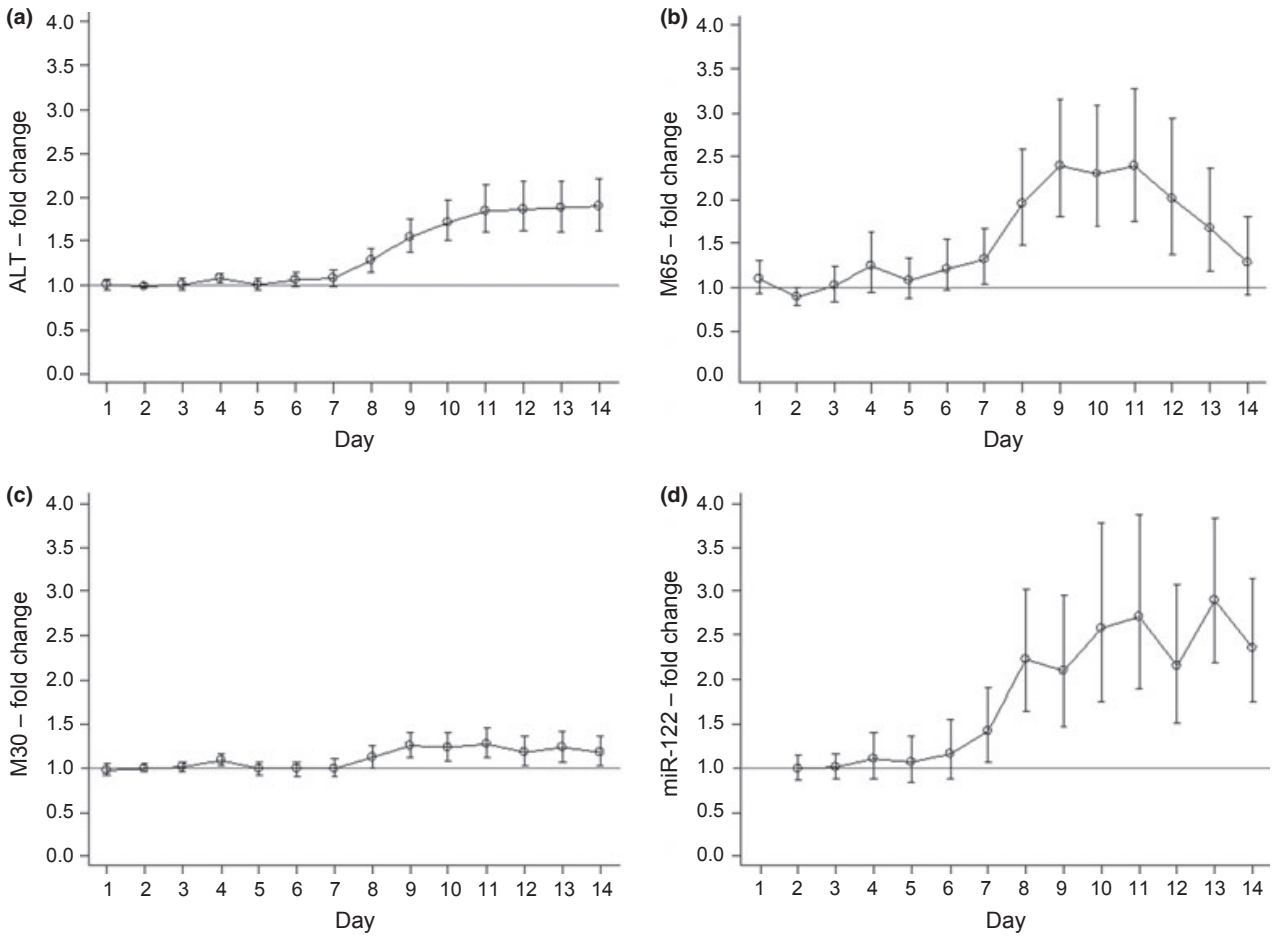


**Fig. 1.** Serum measurements of liver injury biomarkers in the acetaminophen study. (a) alanine aminotransferase (ALT); (b) full-length K18 (M65); (c) cleaved K18 (M30); (d) microRNA-122 (miR-122) are depicted as relative differences compared to baseline ± 95% confidence limits. Baseline levels were defined as the average of the values of the first 3 days (average of day 2–3 for miR-122). Acetaminophen was administered starting on day 4 and ending on day 11. If a confidence interval does not contain the value 1.0, the difference from baseline is statistically significant at the 5% level.

**Table 2.** Pearson correlation coefficients between ALT and liver biomarker values in the acetaminophen study

| | Day 5 | Day 6 | Day 7 | Day 8 | Day 9 | Day 10 | Day 11 | Day 12 | day 13 | Day 14 |
|---|---|---|---|---|---|---|---|---|---|---|
| K18:M65 | 0.22 | 0.15 | 0.30 | **0.60** | **0.68** | **0.79** | **0.79** | **0.70** | **0.78** | **0.79** |
| K18:M30 | 0.09 | 0.20 | 0.08 | **0.50** | **0.60** | **0.57** | **0.69** | **0.66** | **0.66** | **0.56** |
| miR-122 | 0.19 | 0.26 | 0.09 | **0.42** | **0.56** | **0.64** | **0.71** | **0.62** | **0.65** | **0.67** |

K18, keratin-18; miR-122, microRNA-122.
Boldface values = $P < 0.05$.

baseline levels and 22 matched patients without ALT elevations. The miRNA was elevated by 1.4-fold at week 1 ($P = 0.03$) and 1.9-fold at week 8, ($P = 0.01$) and showed a tendency towards significant elevation also at week 2 and 4 (w2:1.4-fold, $P = 0.07$; w4:1.6-fold, $P = 0.06$) (Fig. 2d). The two additional liver biomarker candidates GLDH and AFP were also analysed in the total 76 patients. GLDH was significantly elevated at week 1 (1.5-fold, $P < 0.001$) and week 12 (1.3-fold, $P = 0.03$) and showed a tendency to be higher than baseline at most other time points (w2:1.2-fold, $P = 0.10$; w4:1.2-fold, $P = 0.08$; w8: 1.3-fold, $P = 0.05$) (Fig. 2e). AFP levels were significantly elevated compared to baseline at all time points after treatment was initiated (Fig. 2f). Pearson correlations between ALT and the novel biomarkers was strongest at week 1, with observed correlations of 0.69 for M65, 0.44 for M30, 0.70 for miR-122 and 0.74 for GLDH (Table 4). Alfa-foetoprotein was the only biomarker which did not show any significant correlation with ALT at this time point.

**Table 3.** Median values of liver injury markers in the 76 subjects in the HIV/TB study

| Markers | | Week 0 | Week 1 | Week 2 | Week 4 | Week 6 | Week 8 | Week 12 |
|---|---|---|---|---|---|---|---|---|
| ALT (U/L) | Mdn | 26 | 30 | 38 | 36 | 31 | 33 | 32 |
| | IQR | 19–35 | 23–46 | 26–64 | 25–51 | 22–52 | 23–49 | 26–43 |
| AST (U/L) | Mdn | 30 | 39 | 40 | 36 | 39 | 38 | 36 |
| | IQR | 25–41 | 29–64 | 27–59 | 27–50 | 28–53 | 26–45 | 27–55 |
| ALP (U/L) | Mdn | 108 | 116 | 115 | 111 | 111 | 106 | 110 |
| | IQR | 95–120 | 101–145 | 101–154 | 97–136 | 94–142 | 91–132 | 93–137 |
| TBIL (μmol/L) | Mdn | 3.0 | 3.0 | 4.0 | 4.0 | 4.0 | 3.0 | 4.0 |
| | IQR | 2.0–4.3 | 2.0–5.0 | 2.0–5.0 | 2.5–5.0 | 2.0–6.0 | 2.0–4.0 | 3.0–5.0 |
| CK (U/L) | Mdn | 43 | 29 | 31 | 37 | 58 | 43 | 56 |
| | IQR | 23–63 | 17–56 | 20–64 | 21–59 | 30–82 | 30–74 | 31–89 |
| GGT (U/L) | Mdn | 33 | 37 | 60 | 61 | 47 | 45 | 52 |
| | IQR | 18–66 | 21–75 | 31–127 | 39–117 | 31–113 | 24–114 | 24–88 |
| GLDH (U/L) | Mdn | 4.0 | 5.0 | 5.0 | 5.0 | 4.0 | 4.0 | 5.0 |
| | IQR | 3.0–6.0 | 3.0–11.0 | 3.0–8.0 | 3.0–8.0 | 3.0–6.8 | 3.0–8.0 | 3.0–7.8 |

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TBIL, total bilirubin; CK, creatine kinase; GGT, γ-glutamyltransferase; GLDH, glutamate dehydrogenase; Mdn, median; IQR, interquartile range.

### Evaluation of specificity using subjects with exercise-induced muscular injury

We used exercise-induced muscular injury as a control to assess whether the novel biomarkers were affected by damage to a non-hepatic tissue. Plasma from 12 individuals was collected before and after participation in an extreme adventure race. ALT, AST and CK levels were highly elevated in the plasma of the participants following the race, whereas GLDH remained unchanged. Similarly, neither M65 nor M30 changed after the exercise, whereas the levels of miR-122 were significantly lower in the post-race compared to the pre-race plasma samples. The results are shown in Table 5.

## Discussion

Drug-induced liver injury is an event that has a detrimental impact on drug development and patient safety; therefore the identification of novel translational biomarkers that are both sensitive and specific to the liver would have great benefit (34). The search for novel biomarkers is ongoing: a number of biomarker candidates are now being clinically qualified within the Innovative Medicines Initiative's SAFE-T (Safer and faster evidence-based translation) consortium to determine their clinical value (12). The aim of our study was to assess the K18 markers (M65 and M30), miR-122, GLDH and AFP as novel DILI biomarkers. The potential biomarkers were compared against ALT based on sensitivity (as defined by time to first significant elevation) and specificity (with respect to hepatic origin) and the two human DILI cohorts used in this study were characterized by mild elevations in plasma ALT levels, representative of early development of DILI.

In the acetaminophen study, the total K18 marker M65 was more sensitive in a temporal sense than ALT as it increased significantly already at day 7 compared to ALT, which did not reach significance until day 8. The maximal fold increase levels of M65 was also larger than for ALT (ALT:1.9-fold, M65:2.4-fold). These data are supported by recent observations in patients following acetaminophen overdose (35). In addition, M65 levels dropped towards baseline when therapeutic doses of acetaminophen were no longer administered whereas ALT-levels remained elevated in the study after the treatment was withdrawn, suggesting a shorter half-life in the circulation for M65. The advantage of the faster turn-over is the possibility to determine whether a treatment has any effect and gives the biomarker diagnostic properties. Furthermore, the K18 biomarkers add mechanistic information about the type of cell death, M65 elevations indicating necrosis with additional apoptosis occurring from day 8 onwards detected by M30. In the HIV/TB study, both M65 and M30 were elevated after 1 week of treatment and then went back to baseline. Also in this study, a larger response in M65 compared to M30 could be observed indicating both necrosis and apoptosis ongoing. Earlier studies have shown that both M65 and M30 increase in preclinical studies during acetaminophen-DILI and in human acetaminophen overdose patients (13, 14, 36). Interestingly, our results for M30 are different to a human study of volunteers receiving heparin for 5 days (37). The data from the heparin study also showed transient ALT, M65 and miR-122 levels, however, M30 was not affected by the treatment. The mechanism of insult to the human liver during heparin treatment is unknown but seems to differ from acetaminophen-induced liver injury and HAART/TB-induced liver injury. In contrast to the two DILI cohorts in this study, M65 and M30 did not change in the muscular injury cohort even though ALT was highly elevated, confirming that these novel biomarkers do not increase as a response to muscular injury.

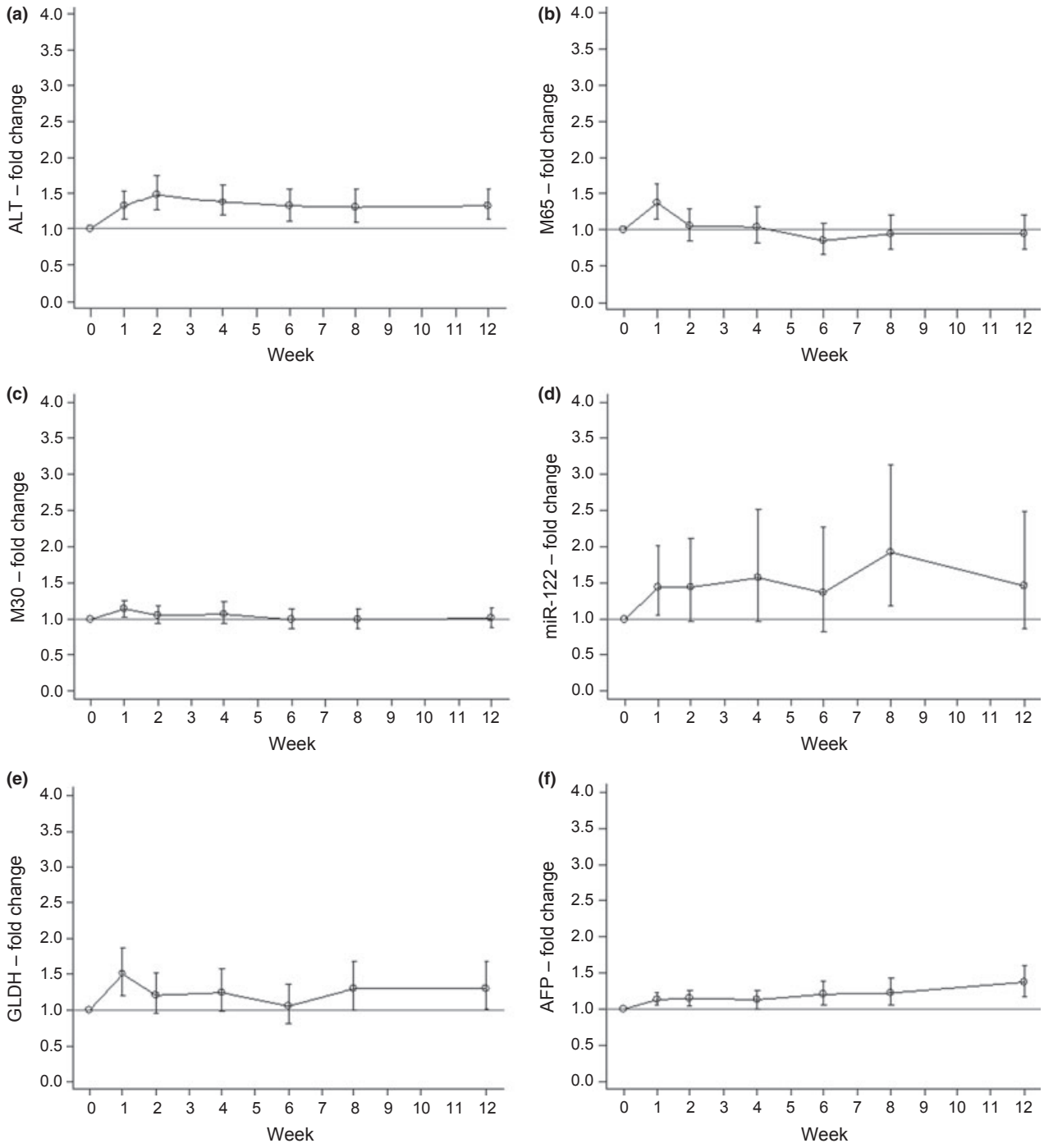


**Fig. 2.** Plasma measurements of liver injury biomarkers in the HIV/TB study. (a) alanine aminotransferase (ALT); (b) full-length K18 (M65); (c) cleaved K18 (M30); (d) microRNA-122 (miR-122); (e) glutamate dehydrogenase (GLDH); (f) alpha-foetoprotein (AFP); are depicted as relative differences compared to week 0 ± 95% confidence limits. Week 0 was drug-free and HIV/TB treatments were initialized from week 1. If a confidence interval does not contain the value 1.0, the difference from baseline is statistically significant at the 5% level.

An additional advantage of the K18 markers as biomarkers for DILI is their long stability in plasma after sampling (38).

In the acetaminophen study, miR-122 was elevated, confirming the results from earlier studies of acetaminophen-induced liver injury in rodents as well as in humans (15, 16, 39, 40). Just like M65, miR-122 was an earlier indicator of reporting liver injury than ALT as it was elevated already at day 7, which is in agreement other studies reporting miR-122 to increase earlier in liver

**Table 4.** Pearson correlation coefficients between ALT and liver biomarker values in the HIV/TB study

| | Week 0 | Week 1 | Week 2 | Week 4 | Week 6 | Week 8 | Week 12 |
|---|---|---|---|---|---|---|---|
| K18:M65 | **0.42** | **0.69** | **0.57** | **0.56** | **0.42** | **0.35** | **0.37** |
| K18:M30 | 0.19 | **0.44** | 0.20 | **0.26** | 0.19 | **0.29** | 0.21 |
| miR-122 | **0.52** | **0.70** | **0.35** | **0.47** | 0.34 | **0.38** | 0.18 |
| GLDH | **0.44** | **0.74** | **0.46** | **0.45** | **0.40** | **0.52** | **0.47** |
| AFP | 0.08 | 0.31 | 0.08 | 0.13 | 0.11 | 0.07 | 0.14 |

K18, keratin-18; miR-122, microRNA-122; GLDH, glutamate dehydrogenase; AFP, alpha-foetoprotein.
Boldface values = $P < 0.05$.

**Table 5.** Median values of liver injury markers before and after race in the muscular injury study

| Markers | | Pre-race | Post-race | Fold change | *P*-value |
|---|---|---|---|---|---|
| ALT (U/L) | Mdn | 23 | 59 | 2.5 | 0.003 |
| | IQR | 17–29 | 57–83 | | |
| AST (U/L) | Mdn | 31 | 169 | 5.5 | <0.001 |
| | IQR | 26–36 | 114–276 | | |
| ALP (U/L) | Mdn | 64 | 65 | 1.0 | NS |
| | IQR | 51–85 | 57–86 | | |
| TBIL (μmol/L) | Mdn | 5.0 | 11.0 | 2.2 | 0.004 |
| | IQR | 4.0–6.0 | 6.8–16.0 | | |
| CK (U/L) | Mdn | 145 | 3925 | 27 | <0.001 |
| | IQR | 110–182 | 2538–6395 | | |
| GGT (U/L) | Mdn | 18 | 16 | 0.9 | NS |
| | IQR | 14–24 | 13–27 | | |
| GLDH (U/L) | Mdn | 5.0 | 4.0 | 0.8 | NS |
| | IQR | 3.0–6.0 | 3.0–5.0 | | |
| K18:M65 (U/L) | Mdn | 41 | 37 | 0.9 | NS |
| | IQR | 27–69 | 20–48 | | |
| K18:M30 (U/L) | Mdn | 152 | 139 | 0.9 | NS |
| | IQR | 141–185 | 124–224 | | |
| miR-122 (rel. Qty $\times 10^{-3}$) | Mdn | 2.0 | 0.6 | 0.3 | 0.004 |
| | IQR | 1.0–3.0 | 0.4–1.0 | | |

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TBIL, total bilirubin; CK, creatine kinase; GGT, γ-glutamyltransferase; GLDH, glutamate dehydrogenase; K18, keratin-18; miR-122, microRNA-122; rel. Qty, relative quantity; Mdn, median; IQR, interquartile range; NS, not significant.

damage than ALT (15, 38–41). Furthermore, the maximal relative increase of miR-122 compared to baseline in this study was larger than for ALT (ALT:1.9-fold; miR-122:2.9-fold) further demonstrating the enhanced utility of miR-122 as a DILI biomarker. It is not known whether the earlier detection of miR-122 is because the miRNA is released into the plasma at an earlier time point than ALT in an active process or whether it is a result of the more sensitive method of detection (qRT-PCR, real-time quantitative reverse-transcription PCR). At the last day of our acetaminophen study, the miRNA returned towards baseline whereas ALT remained high, which corresponds with that the half-life of miR-122 in plasma has been described to be shorter than for ALT (16). In the HIV/TB study, miR-122 was elevated to the same extent as ALT after 1 week of treatment and showed a tendency towards significantly higher levels throughout the study. Since all evaluated biomarkers were elevated already at the first time point measured in the HIV/TB study (week 1), analysis of miR-122 at earlier time points would have been interesting. It should also be emphasized that the power of the miR-122 analysis was weaker than for the other biomarkers in the HIV/TB study, since a smaller subset was analysed for miR-122. Even though miR-122 was the most sensitive (with respect to time of first elevation and fold increased from baseline) biomarker in our two DILI studies, the increased sensitivity of a 1000-fold described elsewhere was not reached (16). This could be explained by the lower degree of liver injury in our cohorts compared to acetaminophen overdose/acute liver injury patients. In the muscular injury study, miR-122 did not increase, which confirms the origin of ALT was not hepatic and the enhanced organ specificity of miR-122 as demonstrated elsewhere (41, 42). Apart from the sensitivity and organ specificity of miR-122, there are additional advantages to using this miRNA as a biomarker for DILI. mir-122 is very abundant and stable in plasma and can be detected using small amounts of starting material (20). Also, miR-122 is evolutionary conserved between species, which makes it possible to use the same assay for detection in preclinical species as well as in the clinical setting. Furthermore, miR-122 has been

shown to correspond better to liver histopathological results than ALT (41, 42).

The potential DILI biomarkers GLDH and AFP were only analysed in the HIV/TB study, because of a lack of available sample volumes in the acetaminophen study. GLDH was elevated at the first week of the HIV/TB study and showed a tendency towards elevation throughout the study. These data are consistent with the fact that GLDH levels are elevated in NAFLD and hepatitis C patients and during liver injury caused by heparin or an acetaminophen overdose (17, 35, 37). In contrast, GLDH levels remained unaffected in the muscular injury study, further supporting its usefulness as a liver-specific translational biomarker in DILI. AFP was slightly elevated after treatment at all time points in the study, which indicated that the low extent of DILI was enough to stimulate the expression of this marker of cell regeneration. AFP was not measured in the muscular injury study, again because of a lack of available sample volumes. AFP is also elevated during chronic liver disease and in response to chemotherapeutic agent efficacy during hepatocellular carcinoma (HCC) (43). This is a rapidly evolving field and to date there are still no defined reference intervals of these investigational biomarkers in human disease populations, particularly in patients with HCC who might be treated with cytotoxic agents resulting in increased levels of liver-derived biomarkers. Therefore, for the current investigation we have chosen to exclude this from our analysis so that changes in biomarker profiles can be investigated during the acute phase of liver injury and were attributed to toxic drug action, and not to to chemotherapeutic agent efficacy.

Except for AFP, the correlations of the novel biomarkers with ALT in both our DILI studies were significant when ALT levels were elevated, indicating that there was an overlap of subjects demonstrating elevations in ALT and in the novel biomarkers. However, it is important to point out that a perfect correlation with ALT was not expected, since the new biomarkers were selected for qualification based on the properties divergent to ALT. Our results indicate that even though the new biomarkers did correlate with ALT, there were interindividual differences in the biomarker profiles between the subjects in each study. The absence of correlation between AFP and ALT in HIV/TB study might be explained by the fact that AFP is a marker of regeneration/proliferation and not an injury marker like ALT.

One major drawback of both of our DILI studies is the fact that the patients have been selected based on ALT levels, which also is the endpoint that the novel biomarkers are evaluated against. How to evaluate biomarker data without selecting for ALT is a problem, since ALT is considered the gold standard biomarker when no histopathology data are available. When dividing patients into subgroups based on the levels of ALT-expression, we might fail to spot out biomarkers with diverse properties to ALT. To exemplify the selection problem, the subjects of the APAP study were analysed as two subgroups based on ALT levels; 'responders' (ALT elevation >2 × baseline) and 'non-responders' (ALT elevation <1.5 × baseline) as described in Winneke *et al.* (30). ALT levels then increased earlier in the responders compared to the novel biomarkers investigated in the study. However, if the M65-values were used to define responders/non-responders, M65 turned out to be the most sensitive biomarker (data not shown). Thus, to counteract the bias in favour of ALT in our studies, we decided not to group the individuals after ALT levels but to analyse all the individuals in each study as one large group. However, 50% of the subjects in both studies were still selected based on high plasma ALT levels and it is therefore not surprising that ALT comes out well in comparison to other biomarkers. Regardless of this selection bias, ALT was not the best biomarker in the acetaminophen study, whereas it performed better than other biomarkers in the more heterogenous HIV/TB study. A drawback of including all the subjects in the analysis is that the ALT elevations (and elevations of biomarkers which are highly correlated with ALT) become smaller than if only the individuals with high ALT levels would have been included. However, this way of analysis makes the conditions more equivalent for all the biomarkers analysed and it is the comparison between the elevations of the biomarkers which are important and not the magnitude *per se*. We stress that caution should be taken when correlating novel biomarker data with ALT, considering ALT as the golden standard.

Drug-induced liver injury is a rare event and it is difficult to access well-characterized cohorts covering the time-course of the injury (2). Hence, there are several discrepancies between our two DILI studies which might explain the different results obtained in the biomarkers analysed. The acetaminophen cohort was a highly controlled study where healthy volunteers received the same amount of acetaminophen at fixed time points in an identical environment, which is a reason for more clear cut results in this study. In comparison, the HIV/TB cohort contained patients with low body-weights (mean BMI = 20), sick to different degrees in HIV and/or TB. In addition, the participants in the study received different combinations of medical treatments depending on whether they were diagnosed only with HIV, TB or both. As a result, the individual ALT peak evaluations were observed at different time points in this study. Thus, none of the effects in the HIV/TB study were very large. Just like the acetaminophen study, the muscular injury study consisted of healthy individuals, without any signs of liver abnormalities. The increase in plasma bilirubin seen in this study has been described after extreme exercise because of increased haemolysis (44). Intake of ibuprofen by the athletes during the race might also explain the change in this parameter, since NSAIDs compete for the same transporters into the liver as bilirubin (45, 46). However, the stable levels of GLDH,

the high values of CK and the ratio of AST/ALT >1 indicate that the ALT elevations are because of muscular damage, in this study (47).

The occurrence of asymptomatic elevations of liver function tests during clinical trials in drug development occur frequently and may not be drug-related but reflect other factors, such as exercise (48) and diet (49). The underlying mechanisms of those elevations are to a large extent unknown, but a study on healthy volunteers identified weight lifting and probably other types of muscular training as causes of ALT elevations (6). Subjects studied in Phase I clinical trials are often young healthy volunteers who in their normal life perform some kind of recreational exercise, and during outpatient trials the volunteers usually continue with their normal life, including exercise. More liver-specific markers, such as miR-122 and K18, would help to identify the origin of ALT elevations and would help to interpret early signals relating to liver safety concerns. Limitations of K18 and miR-122 as biomarkers for DILI are that they are elevated during other types of liver diseases, such as liver steatosis/non-alcoholic steatohepatitis, fibrosis and in hepatitis B and C (41, 50–52).

Taken together, the two DILI studies show similar results, both demonstrating that M65 and miR-122 are biomarkers competing with ALT in terms of sensitivity, as defined as an earlier indicator of liver injury, and additional mechanistic information is gained by M30 measurement. Furthermore, these novel biomarkers outperform ALT when it comes to specificity as defined as of organ of origin and stability in serum/plasma after sampling and their short half-life in the circulation also makes them valuable as diagnostic biomarkers. Hence, using M65 and miR-122 as a complement to ALT would help to explain undesirable elevations in ALT in preclinical/clinical studies, either as a tool to exclude DILI when ALT is not released from the liver or as a relevant DILI signal when other reasons for ALT elevation can be excluded by the additional information gained from M65 and miR-122. Within this current investigation, we have defined sensitivity in the temporal sense of an earlier reporter of liver injury and specificity relating to organ of origin. It is currently beyond the scope of the current investigation to determine the ability of a biomarker to correctly identify individuals who do not have toxicity and to assess the ability of a biomarker to correctly identify toxicity when it occurs. However, this current clinical data provide evidence that these biomarkers hold the potential to begin to investigate these questions and to determine the value of these biomarkers in wider cohorts of DILI investigated prospectively as currently performed within the IMI SAFE-T consortium.

## Acknowledgements

We thank Elsa Mesfin, Maarit Bäcklander and Gunilla Sjelvgren for excellent technical assistance.

*Financial support*: This work was supported by research grants from European and Developing Countries Clinical Trial Partnership (grant number CG_TA.05.40204_005, CT.2005.32030.001), the Swedish Research Council (grant number 348-2011-7383) and the National Center for Advancing Translational Sciences, National Institutes of Health (Grant Award Number UL1TR000083). Authors B. K. P and D. J. A. would like to acknowledge financial support from the Medical Research Council through the Centre for Drug Safety Science (grant number G0700654). D. J. A. would also like to acknowledge additional support from a Wellcome Trust Research Fellowship.

*Conflict of interest*: The authors do not have any disclosures to report.

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